Mechanism of Cholesterol and Phosphatidylcholine Exchange or Transfer between Unilamellar Vesicles†

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ABSTRACT: The mechanism of cholesterol and phosphatidylcholine exchange has been investigated by following the transfer of radiolabeled cholesterol and phosphatidylcholine from negatively charged, unilamellar cholesterol–egg yolk phosphatidylcholine donor vesicles to neutral acceptor vesicles of similar composition. Vesicles were incubated in the absence of protein and were stable to fusion over the course of the experiment. At intervals, donor and acceptor vesicles were separated by passage through a column of DEAE-Sepharose; <1% of the charged and 80–95% of the neutral vesicles were recovered in the eluate. Over 12 h at 37 °C, 90% of the donor vesicle [4-^{14}C]cholesterol was transferred to the acceptor vesicles in a first-order process whose half-time was 2.3 ± 0.3 h. This indicates that transfer of cholesterol molecules from the inner to outer monolayer of the vesicle bilayer is not rate limiting in exchange. In contrast to cholesterol exchange, the half-time for 1-palmitoyl-2-oleoyl[1-^{14}C]phosphatidylcholine exchange was 48 ± 5 h so that more than six molecules of cholesterol were transferred for each molecule of phosphatidylcholine. The interfacial flux of cholesterol from the donor bilayer is 5.3×10^{-15} mol cm^{-2} s^{-1} (~3 molecules/min for an average vesicle) and is similar to fluxes observed in other systems where phosphatidylcholine or cholesterol ester exchange is catalyzed by an exchange protein. When the acceptor vesicle concentration was increased 20-fold in cholesterol exchange experiments or 9-fold in phosphatidylcholine exchange experiments, the rate of label transfer was not affected. The activation energy of cholesterol exchange between 15 and 37 °C was 73 ± 5 kJ mol^{-1}. Transfer of cholesterol across a dialysis membrane is shown to be a slow process whose rate may be predicted by application of Fick's first law of diffusion. These results are only consistent with a mechanism of lipid exchange in which cholesterol and phosphatidylcholine diffuse through the aqueous phase; the experimental activation energy is associated with desorption of lipid from the donor bilayer into the aqueous phase.

An understanding of the mechanisms involved in the transport of cholesterol from tissues to plasma and its subsequent transfer to the liver for degradation is important in developing a model for the reversal of atherosclerosis. A significant aspect of this transport process is the exchange or transfer of cholesterol among lipoproteins and cell membranes. Recent investigations have shown that lipid exchange may proceed in the absence of metabolic energy and does not require specific protein or ionic interactions [for reviews, see Bruckdorfer & Graham (1976), Bell (1978), and Smith & Scow (1979)]. However, numerous complications arise in the investigation of lipid exchange in biological systems due to protein and lipid transfer, adsorption of vesicles to membranes, and the complex structures of biological membranes and lipoproteins (Jonas & Maine, 1979; Giraud & Claret, 1979).

In an appropriate model system, these complications may be minimized so that in the absence of fusion one of two limiting mechanisms may operate in lipid exchange: (1) lipid molecules diffuse through a complex formed by the transient fusion of two lipid monolayers or bilayers following collision of the two particles ("collision complex"; Gurd, 1960) or (2) free lipid molecules diffuse through the aqueous phase separating the donor and acceptor particles (Hagerman & Gould, 1951). There is no general agreement on which of these two mechanisms is operative for cholesterol and phosphatidylcholine exchange in biological systems or model membranes. Smith and co-workers (Charlton et al., 1976, 1978; Kao et al., 1977; Doody et al., 1980) and Roseman & Thompson (1980) have established that aqueous diffusion operates for exchange of fluorescent lipid molecules. On the other hand, phospholipid exchange experiments involving phosphatidylcholine bilayer vesicles have been interpreted in terms of either an aqueous diffusion mechanism (Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Thilo, 1977; Duckwitz-Peterlein & Moraal, 1978) or a mechanism involving collisions between the lipid vesicles (Kremer et al., 1977).

The mechanism of cholesterol exchange between phospholipid vesicles has not been determined although the phenomenon has been well established (Haran & Shporer, 1977; Nakagawa et al., 1979; Backer & Dawidowicz, 1979). Previous studies of cholesterol exchange between vesicles have been complicated by the presence of albumin which is required to maintain the stability of the vesicles and aid in their separation (Nakagawa et al., 1979; Backer & Dawidowicz, 1979). The presence of apolipoproteins in some commercial preparations of albumin may also complicate these experiments (Fainaru & Deckelbaum, 1979).

This investigation utilizes well-characterized, unilamellar cholesterol–phosphatidylcholine vesicles to study the mechanism of cholesterol and phosphatidylcholine exchange in a model membrane system. So that the effect of protein on exchange was minimized, a method for separation of the vesicles has been used which does not require protein to maintain the stability of the vesicles or for their efficient separation. Radiolabeled lipids were used to most closely mimic the structure of the unmodified molecules in order to avoid the complications arising from addition of reporter groups. The kinetics and activation energy of exchange are analyzed in terms of a model whose rate-limiting step is the desorption of lipid from the vesicle bilayer into the aqueous phase.

Experimental Procedures

Materials

Cholesterol and oleic anhydride were used as supplied by Sigma Chemical Co. (St. Louis, MO). Dicetyl phosphate was obtained from Albright and Wilson (Birmingham, United

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Kingdom), and taurocholic acid and egg yolk phosphatidylcholine were used as supplied by Calbiochem-Behring Corp. (La Jolla, CA). The lipids were stored under N_2 at 4 °C as stock solutions in chloroform–methanol (1:1). The concentration of PC[1] in stock solutions was determined from inorganic phosphorus (Sokoloff & Rothblat, 1974). [7-^{3}H(N)]Cholesterol (sp act. 23 Ci/mmol), [4-^{14}C]cholesterol (sp act. 55 mCi/mmol), cholesteryl [1-^{14}C]oleate (sp act. 51 mCi/mmol), and [^{3}H(G)]taurocholic acid (sp act. 3.4 Ci/mmol) were purchased from New England Nuclear (Boston, MA). 1-Palmitoyl-2-oleoyl[1-^{14}C]phosphatidylcholine (100 mCi/mmol) was purchased from Applied Science Laboratories, Inc. (State College, PA). [7-^{3}H(N)]Cholesteryl oleate was prepared to a specific activity of 55 mCi/mmol by reaction of oleic anhydride and [7-^{3}H(N)]cholesterol (the specific activity was reduced by mixing with unlabeled cholesterol in chloroform) according to the procedure of Lentz et al. (1975).

The purity of egg yolk phosphatidylcholine, cholesterol, and the labeled lipids was assayed by thin-layer chromatography on Silica Gel G in three solvent systems: (1) petroleum ether–diethyl ether–acetic acid (75:24:1 v/v/v) (2) chloroform–methanol–water (65:25:4 v/v/v), and (3) benzene–ethyl acetate (60:40 v/v). The purity of dicetyl phosphate was assessed by chromatography in chloroform–methanol–ammonium hydroxide (56:18:3 v/v/v). Lipids were visualized by spraying developed thin-layer plates with 50% sulfuric acid and charring at 200 °C for 15 min. The distribution of radioactivity was determined by scraping the plates, at positions conforming to standards, into liquid scintillation vials and analyzing for radioactivity by liquid scintillation counting in 10 mL of scintillation cocktail (ScintiVerse, Fisher Scientific Co.). Application of 100 μg of unlabeled lipid gave a single spot by charring. Before incubation, all radiolabels were >97% pure. After incubation of [4-^{14}C]cholesterol-containing unilamellar vesicles for 12 h at 37 °C >95% of the labeled cholestrol cochromatographed with a cholesterol standard. Ethanol was redistilled before use.

Methods

Preparation of Unilamellar Vesicles. Vesicles were prepared by the method of Barenholz et al. (1977). Aliquots of the appropriate lipid stock solutions were mixed and dried under N_2 to a thin film on the walls of a test tube. Butylated hydroxytoluene (0.01% w/w of egg PC) was included to reduce oxidation. Traces of remaining solvent were removed by drying for 2 h at 40 °C under vacuum. The dry lipid mixtures were taken up in either 20 mM sodium phosphate buffer, pH 6.0, or 0.15 M NaCl and 20 mM sodium phosphate buffer, pH 7.0, to a final lipid concentration of <10 mg/mL and a total volume between 4 and 10 mL. The lipid was dispersed by sonication under N_2 at 4 °C with a Branson Sonifier Model 350 at setting 4 and tapered microtip immersed 2 cm into the solution. The solution was sonicated for 30 min in 5-min intervals separated by 2-min cooling periods. Multilamellar particles, undispersed lipid, and titanium were sedimented by centrifugation at 40000 rpm (139700*g* max) for 2 h in a Beckman Type 65 rotor. The clear supernatant was stored under N_2 at 4 °C and used within 1 week of preparation.

Preparation of Bile Acid–Phosphatidylcholine Micelles. Micelles were prepared according to the procedure of Mazer et al. (1980). A mixture of 0.5 mol of egg yolk phosphatidylcholine/mol of taurocholic acid was prepared in ethanol with or without [^{3}H(G)]taurocholic acid. The solvent was evaporated under a stream of N_2 and dried to constant weight under a vacuum at 40 °C for 24–48 h. Then 0.15 M and 20 mM sodium phosphate buffer, pH 7.0, was added to give a final lipid concentration of 6.25 mg/mL. The lipid was dispersed by vortexing vigorously for 2 min and left to stand at 4 °C under N_2 for 2 days with periodic vortexing.

Separation of Vesicles. Two populations of vesicles, negatively charged "donor" vesicles and neutral "acceptor" vesicles, were separated by charge on ion-exchange columns by a method based on the procedure of Hellings et al. (1974) as modified by van den Besselaar et al. (1975). Vesicles were prepared as described above in 20 mM sodium phosphate buffer, pH 6.0. Donor vesicles contained 1.0 μCi of [4-^{14}C]cholesterol or 1-palmitoyl-2-oleoyl[1-^{14}C]phosphatidylcholine/mg of total vesicle lipid in exchange experiments or 0.1 μCi of cholesteryl [1-^{14}C]oleate/mg of total vesicle lipid in control experiments. The final donor vesicle composition was 20 mol % cholesterol, 65 mol % phosphatidylcholine, and 15 mol % dicetyl phosphate. Acceptor vesicles comprised 80 mol % egg yolk phosphatidylcholine and 20 mol % cholesterol; 0.1 μCi of [7-^{3}H(N)]cholesteryl oleate/mg of total vesicle lipid (0.1% w/w) was added as a nonexchangeable marker to monitor recovery. Incubations were carried out in 20 mM sodium phosphate buffer, pH 6.0, at total vesicle lipid concentrations from 0.4 to 9.0 mg/mL; temperature was regulated within 0.2 °C by incubation in a Forma Model 2095 water bath. Acceptor vesicles were in at least 10-fold excess in all incubations with donor vesicles to minimize back exchange of label.

A total of 100 mL of DEAE-Sepharose CL-6B (Pharmacia Fine Chemicals) was washed 3 times with a 5-fold excess of 20 mM sodium phosphate buffer, pH 6.0. The buffer was decanted off, and the gel was diluted 1:1 (v/v) with the buffer. Small columns (~1 cm in length) of ion-exchange resin were prepared by pouring 1.0 mL of the diluted DEAE-Sepharose into 9 in. Pasteur pipets plugged with cotton. A total of 1.0 mL of the sodium phosphate buffer was applied to the column, and the eluate was discarded. A total of 0.25 mg of sonicated egg yolk phosphatidylcholine vesicles in 0.1 mL of buffer was applied to the columns to reduce nonspecific adsorption and improve recovery of acceptor vesicles. This eluate was also discarded. Directly following this, a 0.25-mL aliquot of the incubation mixture was applied to the column, allowed to enter the column completely, and immediately eluted with 1.00 mL of the sodium phosphate buffer; elution was complete in 2 min (possible transfer of label to the column is negligible over 2 min at room temperature). This eluate was collected directly into liquid scintillation vials (MiniVials, Fisher Scientific Co.), and the samples were analyzed by liquid scintillation counting of ^{3}H and ^{14}C in 4 mL of liquid scintillation cocktail (ScintiVerse, Fisher Scientific Co.) with a Beckman Model LS 7500. Overlap of ^{14}C into the ^{3}H channel averaged 9%; ^{3}H counts were corrected by using the ^{3}H/^{14}C ratio of a standard which consisted of 0.1 mg of donor vesicles in 1.25 mL of buffer and 4 mL of ScintiVerse. All samples and standards were quenched equally as judged by a ^{137}Cs external standard.

Typically, <1% of the charged vesicles were eluted at zero time. After a 12-h incubation of charged vesicles labeled with cholesteryl [1-^{14}C]oleate and neutral vesicles labeled with [7-^{3}H(N)]cholesteryl oleate, 3–4% of the ^{14}C counts were recovered in the eluate. This corresponds to the small amount of fusion which occurred during incubation (Figure 1). In a

[1] Abbreviations used: PC, phosphatidylcholine; SEM, standard error of the mean; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; PMCA, 3-pyrene methyl ester of 3β-hydroxy-22,23-dinor-5-cholenic acid; LDL, low-density lipoproteins; HDL, high-density lipoproteins; DEAE, diethylaminoethyl.

separate experiment investigating the recovery of vesicles in the eluate as a function of the mole fraction of dicetyl phosphate, 3–4% leakage of vesicles occurred when the mole fraction of dicetyl phosphate was 14%. Since the initial content of dicetyl phosphate is 15% in our experiments, the transfer of dicetyl phosphate is negligible over the course of a typical exchange experiment. With preequilibrated columns, 80–95% of the neutral vesicles were recovered in the eluate at all time points up to 12 h. This is a significant improvement over the recoveries (40–70%) reported by van den Besselaar et al. (1975) and recoveries obtained with nonpreequilibrated columns (50–70%).

Dialysis Experiments. SpectraPor 6 dialysis membranes (Spectrum Medical Industries Inc., Los Angeles, CA) of 50000 mol wt cutoff (estimated pore diameter 7 nm) were rinsed and brought to the boil in deionized water. The tubing was cut into a single thickness and rinsed extensively in deionized water. This membrane was used to separate two chambers of an equilibrium dialysis cell (Fisher Scientific Co.). One compartment was filled with a donor solution of either 20 mol % cholesterol and 80 mol % egg yolk phosphatidylcholine unilamellar vesicles, labeled with 0.1 μCi of [4-^{14}C]-cholesterol/mg of total lipid at a concentration of 1 mg of total lipid/mL of solution, or bile acid–phosphatidylcholine micelles, labeled with 0.5 μCi of [^{3}H(G)]taurocholic acid/mg of total lipid at a concentration of 6.25 mg of total lipid/mL of solution. The second compartment was filled with an identical unlabeled solution. In both sets of experiments a 0.15 M NaCl and 20 mM sodium phosphate buffer, pH 7.0, was used. The dialysis cell was incubated at 37 °C in an environmental room and stirred with a magnetic stirring bar so that complete mixing of the dialysis cell contents was accomplished. Four 10-μL aliquots from each compartment were taken at intervals, and the samples were analyzed by liquid scintillation counting in 4 mL of liquid scintillation cocktail (ScintiVerse, Fisher Scientific Co.). Adsorption of vesicles to the dialysis membrane in these experiments was ruled out by sampling from both chambers of the dialysis cell. At all time points taken for analysis the sum of counts from both chambers remained constant and equal to 100% of the original counts. In addition, 10 μCi of $^{3}H_2O$ added to one chamber at the end of the dialysis experiment equilibrated between the two chambers in <30 min.

Electron Microscopy. Bile acid–phosphatidylcholine micelles were dried on a Formvar-coated grid at 37 °C and negatively stained with 1% uranyl acetate. Unilamellar vesicles were stained with 2% sodium phosphotungstate. Both preparations were observed at 80000–160000× magnification with a Zeiss 10 high-resolution transmission electron microscope operating at 80 kV.

Kinetic Analysis. Kinetic data were analyzed according to the method of McKay (1938) for an isotope-exchange reaction of the type AX + BX* $\rightleftharpoons$ AX* + BX, where A represents the acceptor, B the donor, X the unlabeled species, and X* the labeled species. In experiments where the vesicles are separated on DEAE-Sepharose columns, the fractional transfer of label, x, at time, t, is given by

$$x = \frac{(^{14}C/^{3}H)_t - (^{14}C/^{3}H)_0}{(^{14}C/^{3}H)_{mix}} \quad (1)$$

where $(^{14}C/^{3}H)_t$, $(^{14}C/^{3}H)_0$, and $(^{14}C/^{3}H)_{mix}$ represent the ratios of ^{14}C to ^{3}H in the eluate at time t, at time 0, and in the incubation mixture, respectively. This equation corrects the transfer of ^{14}C label for the recovery of vesicles in the eluate and for the ~1% leakage of donor vesicles at 0 time. For dialysis experiments only a single label is used so that

$$x = \frac{(cpm)_t - (cpm)_0}{(cpm)_{donors}} \quad (2)$$

where $(cpm)_t$ and $(cpm)_0$ are the counts per minute in the originally unlabeled compartment at time t and at time 0, respectively, and $(cpm)_{donors}$ are the counts per minute of the originally labeled compartment at zero time.

Kinetic data may be linearized by introducing R as a rate constant which is a general function of the donor concentration, b, and the acceptor concentration, a

$$-\ln\left(1 - \frac{x}{x_\infty}\right) = Rt\left(\frac{a+b}{ab}\right) \quad (3)$$

where x_∞ is the fractional transfer at infinite time (McKay, 1938). A pseudo-first-order rate constant k may be obtained by setting $R = kb$. This gives the linear expression

$$-kt = \left(\frac{a}{a+b}\right)\ln\left(1 - \frac{x}{x_\infty}\right) \quad (4)$$

which corrects for back exchange of label from acceptors to donors.

Mass exchange is assumed so that an equal distribution of label in donor and acceptor vesicles at infinite time is expected and $x_\infty = a/(a+b)$. The percent equilibrium transfer is then $100 \times x_\infty$. The slope of $\ln(1 - x/x_\infty)$ vs. t was determined by a least-squares procedure. The pseudo-first-order rate constant was calculated from the slope by using $k = -\text{slope} \times a/(a+b)$. Half-times were determined from the relationship $t_{1/2} = (\ln 2)/k$.

Equation 4 describes isotope exchange which follows first-order kinetics regardless of the mechanism of the exchange process (McKay, 1938). Differentiation between true first- and second-order kinetic processes can be accomplished by varying the concentration of the acceptor species. For a second-order process where the second-order rate constant is k_2, $R = k_2ab$. As pointed out above $R = kb$, so that the pseudo-first-order rate constant $k = k_2a$ is directly proportional to the acceptor vesicle concentration, a. In contrast, in a true first-order process with rate constant k_1, $R = k_1b$, indicating that the rate constant is independent of acceptor vesicle concentration.

Experimental fluxes were calculated from $J_o = kcV/A$, where k is the pseudo-first-order rate constant for exchange, c is the concentration of exchangeable lipid in the donor vesicles, V is the volume of the incubation medium, and A is the surface area over which the flux is calculated. In dialysis experiments the theoretical flux of lipid was estimated by Fick's first law of diffusion, $J_t = D_m\Delta c/H$ (Jacobs, 1967), where D_m is the diffusion coefficient of the solute in the membrane, Δc is the concentration gradient across the membrane, and H is the thickness of the dialysis membrane (2×10^{-3} cm). The maximum flux was estimated by assuming that D_m is identical with the free diffusion coefficient of cholesterol calculated according to the Stokes–Einstein equation, $D = kT/6\eta r$ (Jacobs, 1967). Here k is the Boltzmann constant, T is temperature, η is the viscosity of the liquid medium, and $2r$ is the long axis length of the diffusing species. A value of $2r = 1.7$ nm was used for both cholesterol (Shieh et al., 1977) and sodium taurocholate giving $D = 2.7 \times 10^{-6}$ cm^2 s^{-1}. The concentration gradient across the membrane was derived by assuming that only monomeric lipid transfers across the membrane at its critical micellar concentration, so that $\Delta c = 3 \times 10^{-8}$ M for cholesterol (Haberland & Reynolds, 1973) and $\Delta c = 4 \times 10^{-3}$ M for sodium taurocholate (Carey & Small, 1969).

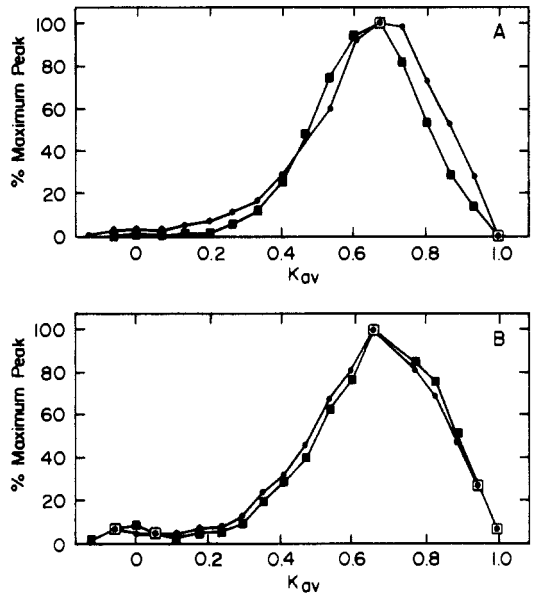

FIGURE 1: Gel filtration patterns of sonicated vesicles before and after 12 h of incubation at 37 °C. Vesicles comprised either 20 mol % cholesterol, 15 mol % dicetyl phosphate, 65 mol % egg yolk phosphatidylcholine, and 0.1 μCi of cholesteryl [1-^{14}C]oleate/mg of lipid (●) or 20 mol % cholesterol, 80 mol % egg yolk phosphatidylcholine, and 0.1 μCi of [7-^{3}H(N)]cholesterol oleate/mg of lipid (■). In (A) vesicles were passed through a 0.9 × 30 cm column of Sepharose CL-2B separately before incubation. In (B) 0.5 mL of a mixture containing 0.1 mg/mL of the charged vesicles and 1.0 mg/mL of the neutral vesicles was applied to the column after incubation for 12 h at 37 °C. Fractions were collected and analyzed for ^{14}C and ^{3}H by liquid scintillation counting.

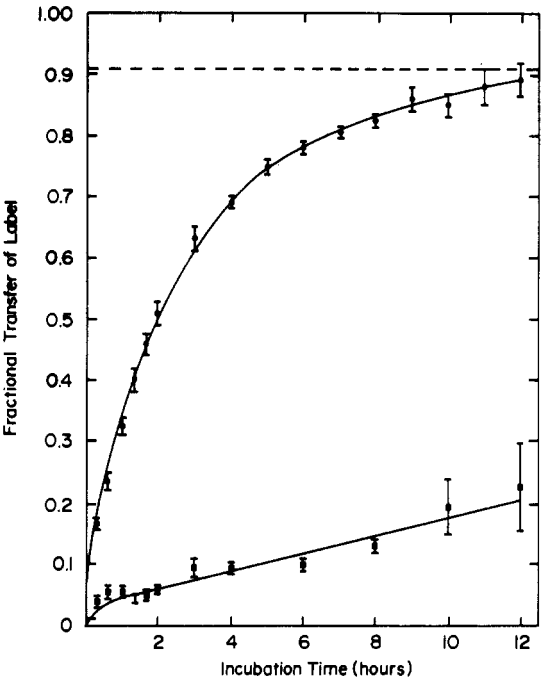

FIGURE 2: Relative rates of cholesterol and phosphatidylcholine exchange at 37 °C. 0.1 mg of donor vesicles/mL was mixed with 1.0 mg of acceptor vesicles/mL and separated on ion-exchange columns as described under Methods. Donor vesicles contained either [4-^{14}C]cholesterol (●) or 1-palmitoyl-2-oleoyl[1-^{14}C]phosphatidylcholine (■). Error bars represent ±SEM for eight [^{14}C]POPC and five [^{14}C]cholesterol experiments. The expected equilibrium transfer of label (---) corresponds to equal distribution of label in donor and acceptor vesicles and is calculated as described under Methods.

Results

In order to assess the stability of the vesicles to fusion, electron microscopy and gel filtration of charged and neutral sonicated vesicles were carried out before and after a 12-h incubation at 37 °C. Before the incubation, the charged and neutral vesicles had similar size distributions on Sepharose CL-2B columns (Figure 1A) with a modal diameter of 23 ± 2 nm as determined by negative-stain electron microscopy. These vesicles were primarily single shelled, and few multilamellar vesicles were evident in negatively stained preparations. After incubation, >90% of the vesicles remained unilamellar as judged by electron microscopy, and <5% of the charged or neutral vesicles appeared in the void volume of a Sepharose CL-2B column (Figure 1B). In addition, the K_{av} for the unilamellar vesicles was not significantly shifted after incubation (cf. Figure 1A,B).

The rate of exchange of the major component of egg yolk phosphatidylcholine, 1-palmitoyl-2-oleoylphosphatidylcholine, and cholesterol between unilamellar vesicles at 37 °C is shown in Figure 2. True mass exchange predicts that at equilibrium label will be distributed equally between donor and acceptor vesicles. The expected equilibrium transfer of label in the experiment of Figure 2 is 90.9% since the acceptor vesicles are in 10-fold excess. This value is nearly reached in a first-order process after 12 h of incubation at 37 °C. This indicates that mass exchange and not mass transfer is operative. The rate of exchange of cholesterol and POPC differs by an order of magnitude with $t_{1/2}$ = 2.3 ± 0.3 h (k = 0.322 ± 0.040 h^{-1}, mean ± SEM, n = 5) and $t_{1/2}$ = 48 ± 5 h (k = 0.016 ± 0.003 h^{-1}, mean ± SEM, n = 8), respectively. These results are in general agreement with other work using unilamellar vesicles (Backer & Dawidowicz, 1979; Roseman & Thompson, 1980; Bloj & Zilversmit, 1977) and with biological systems (Bruckdorfer & Graham, 1976). The interfacial flux of cholesterol from the donor bilayer was 5.3 × 10^{-15} mol cm^{-2} s^{-1} while that for POPC was 8.6 × 10^{-16} mol cm^{-2} s^{-1} (so that for an average vesicle 3 molecules of cholesterol and 0.5 molecule of PC transferred per min).

Table I: Concentration Dependence of Cholesterol Exchange between Unilamellar Vesicles[a]

acceptor vesicle concn (mg/mL)	k (h^{-1})	$t_{1/2}$ (h)
0.4	0.074 ± 0.003	9.5 ± 0.4
0.7	0.076 ± 0.009	9.5 ± 1.0
1.0	0.079 ± 0.014	9.5 ± 1.5
2.0	0.074 ± 0.007	9.6 ± 0.9
5.0	0.067 ± 0.011	11.2 ± 1.7
9.0	0.069 ± 0.009	10.6 ± 1.2
av	0.073 ± 0.004	10.0 ± 0.5

[a] 0.01 mg of donor vesicles/mL was mixed with the indicated concentrations of acceptor vesicles and incubated at 22 °C. At intervals four aliquots were taken for separation on ion-exchange columns as described under Methods. k and $t_{1/2}$ were calculated as described under Methods. Each k and $t_{1/2}$ is the average ± SEM of four experiments. Each experiment consisted of at least six time points.

For determination of the order of lipid exchange with regard to the acceptor vesicles, the rate of cholesterol and POPC exchange was examined over a wide range of acceptor vesicle concentrations. Table I gives the results of a cholesterol exchange experiment in which donor vesicle concentration was kept constant while acceptor vesicle concentration was varied over a more than 20-fold range. There is no significant difference among the six means for the rate of cholesterol exchange (at $p < 0.05$), and the slope of a line relating acceptor vesicle concentration to cholesterol exchange rate is −0.02 ± 0.01 [cf. Nakagawa et al. (1979)]. A similar result was obtained when the acceptor vesicle concentration was varied in

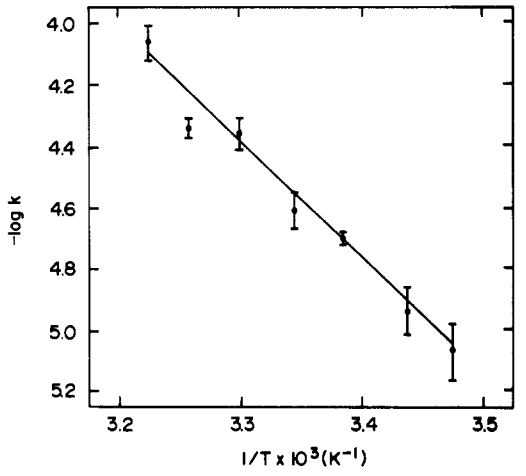

FIGURE 3: Activation energy of 20 mol % cholesterol exchange between 15 and 37 °C. Vesicles were mixed at a concentration of 0.1 mg of donor vesicle lipid/mL and 1.0 mg of acceptor vesicle lipid/mL. Incubations were carried out over a range of temperatures; separation of vesicles was achieved on ion-exchange columns as described under Methods. Error bars represent the mean ± SEM of four experiments at each temperature. Each experimental rate constant was determined from four aliquots taken at six time points.

experiments with [^{14}C]POPC in donor vesicles at a constant concentration of 0.01 mg of lipid/mL. At an acceptor vesicle concentration of 1 mg of lipid/mL, k = 0.016 ± 0.003 h^{-1} (mean ± SEM, n = 3), and at 9 mg of acceptor vesicle lipid/mL, k = 0.010 ± 0.003 (mean ± SEM, n = 3). As with cholesterol exchange, there is no significant difference between these means at $p < 0.05$. Thus cholesterol and phosphatidylcholine exchange between unilamellar vesicles is a first-order process independent of the concentration of acceptor vesicles.

The temperature dependence of cholesterol exchange between unilamellar vesicles comprising 20 mol % cholesterol in egg yolk phosphatidylcholine bilayers is given in Figure 3. The activation energy for this process between 15 and 37 °C is 73 ± 5 kJ mol^{-1} (mean ± SEM, n = 44). This value does not differ significantly (at $p < 0.05$) from the previously published value of 67 ± 4 kJ mol^{-1} for 40 mol % cholesterol exchange (Phillips et al., 1980) and is similar to that reported by Poznansky & Czekanski (1979) for 20 mol % cholesterol–dioleoylphosphatidylcholine vesicles.

The slow rate of dialysis of cholesterol has been cited as evidence in favor of a mechanism of cholesterol exchange that requires close contact between donor and acceptor particles (Quarfordt & Hilderman, 1970; Poznansky & Lange, 1978). The low aqueous phase solubility of cholesterol (Haberland & Reynolds, 1973) may be an impediment to its rapid dialysis since only the aqueous phase monomer is transferred across a dialysis membrane. Table II gives the results of dialysis experiments utilizing three different experimental systems designed to assess the relationship between solubility of lipid monomer in the aqueous phase and the rate of lipid exchange across a dialysis membrane. The diameter of the vesicles (23 nm) and the sodium taurocholate–PC micelles (20 nm) was too great to cross the membrane so that only monomeric lipid was transferred. The rate of dialysis of cholesterol and sodium taurocholate was estimated by application of Fick's first law of diffusion. This relationship is based directly on the aqueous phase solubility of lipids. The similarity between J_t and J_o demonstrates that the rate of dialysis of cholesterol and sodium taurocholate may be predicted from knowledge of their aqueous phase solubilities. Thus, the low solubility of cholesterol prevents its rapid transfer across a dialysis membrane

Table II: Flux of Cholesterol and Sodium Taurocholate across a Dialysis Membrane[a]

system	J_o (mol cm^{-2} s^{-1})	J_t (mol cm^{-2} s^{-1})
taurocholate–PC micelles[b]	6.1 × 10^{-10}	5.4 × 10^{-9}
PC–cholesterol vesicles[c]	2.7 × 10^{-15}	4.0 × 10^{-14}
PC–cholesterol vesicles + 34% v/v ethanol[c,d]	2.6 × 10^{-13}	

[a] J_o (observed flux) and J_t (theoretical flux) were calculated by using the indicated systems as described under Methods at pH 7.0. Each value was determined from four aliquots taken at six time points. [b] Diffusing label was [^{3}H(G)]taurocholate. [c] Diffusing label was [4-^{14}C]cholesterol. [d] Vesicles in ethanol were prepared by adding ethanol to vesicles prepared in 0.15 M NaCl and 20 mM sodium phosphate buffer, pH 7.0.

which presents a macroscopic barrier to diffusion (theoretical $t_{1/2}$ = 38 days). Rapid dialysis of lipid may be achieved by increasing the aqueous phase solubility of cholesterol by addition of ethanol or by using the more soluble sodium taurocholate molecules.

Discussion

Apparent first-order kinetics for cholesterol exchange have been observed in a variety of biological and model systems (Bruckdorfer & Graham, 1976; Smith & Scow, 1979). Since ~67% of the cholesterol in unilamellar vesicles resides in the outer monolayer (Huang et al., 1974) the observation that 90% of the cholesterol in our vesicles is exchanged in a first-order process (Figure 2) indicates that transfer from the inner to outer monolayer of the vesicle bilayer is not a rate-limiting step in cholesterol exchange. A similar result has been obtained by other investigators [Backer & Dawidowicz, 1979; Nakagawa et al., 1979; but see Poznansky & Lange (1978)].

Haran and Shporer (1977) have ruled out fusion as a prerequisite for cholesterol exchange by using a nuclear magnetic resonance procedure. Our gel filtration and electron microscopy results confirm this finding; the extent of vesicle–vesicle fusion is negligible over a 12 h period when compared with the extent of labeled lipid transfer (cf. Figures 1 and 2). In a simple model system which is not complicated by fusion or the presence of protein, two limiting models for cholesterol exchange must be considered. One is a process which involves the transitory mixing of the donor and acceptor vesicle bilayers followed by diffusion of lipid molecules within this collision complex (Gurd, 1960), and the other involves desorption of lipid molecules from the donor bilayer into the aqueous phase where the soluble lipid molecules are free to collide with acceptor vesicles (Hagerman & Gould, 1951). These two models may be differentiated experimentally by considering their kinetic predictions.

Kinetics of Exchange. (1) Collision Complex Model. Kremer et al. (1977) have suggested that phospholipid exchange between PC bilayers is the result of vesicle–vesicle collisions. In such a model, either diffusion within the collision complex or collision frequency may be rate limiting. In either case the relative rates of cholesterol and POPC exchange are not expected to differ since the lateral diffusion coefficients for egg yolk phosphatidylcholine and steroids in bilayers are similar [cf. Kuo & Wade (1979) and Trauble & Sackmann (1972)] and collisions are blind to the lipid transferred. In the case of unilamellar vesicles the rate of cholesterol and POPC exchange differ by more than an order of magnitude (Figure 2).

The expected concentration dependence of lipid transfer by the collision complex mechanism may be determined by comparing the time required for a lipid molecule to transfer from

the donor to the acceptor bilayer by diffusion within the complex to the time required for a collision between a donor and acceptor vesicle. The minimum time required for lipid transfer within the collision complex may be estimated by calculating the time required for cholesterol to diffuse two lattice vacancies (1 nm) within the complex. This time is given by $t = \overline{x^2}/2D$ (Sheludko, 1966), where $\overline{x^2}$ is the mean square displacement of the diffusing molecule and D is its lateral diffusion coefficient. With $D = 1 \times 10^{-8}$ cm^2 s^{-1} (Trauble & Sackmann, 1972), $t = 5 \times 10^{-7}$ s. In contrast, for an elastic collision the time, t taken for a single donor vesicle to collide with an acceptor vesicle is given by $t = 1/(8\pi Drc) = 5.7 \times 10^{-4}$ s/collision [see Sheludko (1966)] where r is the radius of the vesicle (11.5 nm), D is its diffusion coefficient (2×10^{-7} cm^2 s^{-1}; Huang, 1969), and c is the concentration of acceptor vesicles (3×10^{14} particles mL^{-1}). Since diffusion within the collision complex requires only a small fraction of the time between collisions (or alternatively, the frequency of collisions exceeds the frequency associated with transfer of a lipid molecule from the donor bilayer to the acceptor bilayer by 3 orders of magnitude) the rate of lipid exchange by a collision complex mechanism is directly proportional to the concentration of acceptor vesicles in the incubation mixture. Both cholesterol and phosphatidylcholine exchange are independent of the concentration of acceptor vesicles over a wide range of concentrations. These observations indicate that the collision complex mechanism is not an adequate description of lipid exchange between unilamellar vesicles.

(*2*) *Aqueous Diffusion Model.* Although the molecular mechanism of cholesterol exchange in unilamellar vesicles has not been established, fluorescent lipid molecules and phospholipids have been shown to exchange via the aqueous phase (Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Thilo, 1977; Duckwitz-Peterlein & Moraal, 1978; Smith & Scow, 1979; Roseman & Thompson, 1980). The kinetics of lipid exchange in an aqueous diffusion model may be described in a manner analogous to that for the dissolution of a crystal (Moelwyn-Hughes, 1961):

$$\mathrm{VM} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{V} + \mathrm{M} \tag{5}$$

Here VM is the donor vesicle containing lipid, V is the donor vesicle after a lipid molecule has desorbed, and M is the lipid monomer in the aqueous phase. The overall rate of desorption is given by

$$\frac{dq}{dt} = k_1[\mathrm{VM}] - k_2[\mathrm{V}][\mathrm{M}] \tag{6}$$

Here k_1 is the first-order rate constant associated with desorption, [VM] is the total concentration of diffusing lipid in the donor vesicle population per milliliter of incubation medium, and k_2 is a second-order rate constant associated with the collision of monomeric lipid molecules at concentration [M] in the aqueous phase, with donor vesicles at concentration [V] (particles mL^{-1}). At equilibrium, $dq/dt = 0$ and the rate of the desorption step is

$$k_1 = k_2 \frac{[\mathrm{M}][\mathrm{V}]}{[\mathrm{VM}]} \tag{7}$$

Since [V] is constant in the case of POPC and cholesterol exchange (Figure 2), [M]/[VM], which is a reflection of the distribution of diffusing lipid between the aqueous and bilayer phases, is an important parameter in determining the relative rates of cholesterol and POPC exchange [cf. Thilo (1977)]. It is difficult to measure the water solubilities of the sparingly soluble lipids used in this study, but the aqueous phase solubility of monomeric cholesterol [6.5×10^{-8}–7.5×10^{-8} M (Saad & Higuchi, 1965); 2×10^{-8}–4×10^{-8} M (Haberland & Reynolds, 1973; Gilbert et al., 1975)] is much greater than that for monomeric long-chain phosphatidylcholines (4.6×10^{-10} M for dipalmitoylphosphatidylcholine; Smith & Tanford, 1972). For the experimental conditions of Figure 2, [M]/[VM] = 1×10^{-3} for cholesterol and [M]/[VM] = 5×10^{-6} for POPC. Comparison of these two values indicates that the rate of cholesterol exchange will greatly exceed the rate of 1-palmitoyl-2-oleoylphosphatidylcholine exchange by aqueous diffusion. The observations shown in Figure 2 are consistent with this interpretation. In addition, more than six molecules of cholesterol transfer for each molecule of phosphatidylcholine, indicating that cholesterol and phosphatidylcholine exchange proceed independently.

Aqueous diffusion may follow either first- or second-order kinetics depending on the rate-limiting step in exchange. First-order kinetics is predicted when desorption is the rate-limiting step. On the other hand, collisions between lipid molecules (presumed to be present in the water as monomers) and the acceptor vesicle in the adsorption step would proceed at a rate proportional to the concentration of acceptor vesicles. There is no evidence for such a collision mechanism (Table I); rather, the kinetics indicate that exchange is independent of the concentration of acceptor vesicles and that desorption is the rate-limiting step in cholesterol and phospholipid exchange between unilamellar vesicles.

When a dialysis membrane is interposed between the donor and acceptor vesicles, the rate of [^{14}C]cholesterol transfer is reduced by this barrier to diffusion. Here the flux of lipid molecules to the acceptor vesicles is limited by the rate of diffusion across the dialysis membrane rather than desorption from the donor vesicle as observed in solution. Thus, Fick's first law of diffusion is predictive for the rate of cholesterol dialysis (Table II). In the absence of a dialysis membrane, no diffusion barrier is operative so that the maximum flux of lipid molecules from the aqueous phase to the acceptor vesicles accounts for the observed rate of exchange. In a previous report, Phillips et al. (1980) have discussed cholesterol exchange in terms of the barrier to diffusion which arises due to the unstirred water layer associated with a monolayer of tissue culture cells. In this system both desorption and diffusion determine the rate of cholesterol transfer from cells to phospholipid vesicles.

Fluorescent probes have yielded much information on the molecular mechanism of lipid exchange in a variety of systems (Charlton et al., 1976, 1978; Doody et al., 1980; Smith & Scow, 1979; Roseman & Thompson, 1980). Kao et al. (1977) have shown that a fluorescent cholesterol analogue (the 3-pyrene methyl ester of 3β-hydroxy-22,23-dinor-5-cholenic acid) transfers between high-density lipoproteins via the aqueous phase. The rate of exchange of this probe between unilamellar vesicles is similar to that of cholesterol. At 37 °C, $t_{1/2}$ for [^{3}H]PMCA exchange is 31.6 ± 3.2 min (L. R. McLean, M. C. Phillips, and L. C. Smith, unpublished results) while under the same conditions $t_{1/2}$ for cholesterol is 138 ± 18 min. The similarities in rate constants and the mechanism of exchange demonstrate that fluorescent lipid analogues are excellent probes of the mechanism of lipid exchange, but the addition of a fluorescent reporter group can alter the exchange rate.

Molecular Mechanism of Exchange. Figure 4 illustrates the only mechanism of lipid exchange which is consistent with all of the experimental evidence. Here, the rate-limiting step for lipid exchange is the transfer of lipid from the donor bilayer

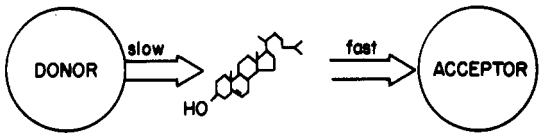

FIGURE 4: Mechanism of cholesterol and phosphatidylcholine exchange and transfer between unilamellar vesicles. Lipid desorbs in a rate-limiting step from the donor bilayer into the aqueous phase. The monomeric lipid molecules have no memory of the vesicle from which they arise and collide with any other vesicle in a random process. Following collision lipid is rapidly adsorbed into the acceptor bilayer. Net mass transfer occurs when the activity of lipid in the donor vesicle population is greater than that of the acceptor vesicle population.

into the aqueous phase. The observed activation energy arises from the energy barrier for desorption. Desorption does not involve a one for one exchange of lipid at a particular site. Mass equilibrium is maintained by adsorption of lipid from the aqueous phase at a separate site in the bilayer. Following transfer of lipid to the aqueous phase, lipid diffuses in the aqueous solution until it collides with another vesicle in a purely stochastic process and is taken up into the bilayer by a rapid adsorption step.

The details of the molecular events associated with desorption can not yet be enumerated, but desorption must involve both the disruption of lipid–lipid interactions in the bilayer and the formation of a cavity in the aqueous phase which will accommodate the diffusing lipid molecule. The rate of lipid exchange, then, will depend both on the energy barrier associated with formation of a lattice vacancy in the bilayer and on the aqueous phase solubility of the lipid. The marked difference in POPC and cholesterol exchange rates in a single bilayer system demonstrates the importance of solubility in determining the rate of lipid exchange. In addition, the activation energy for cholesterol exchange (73 ± 5 kJ mol^{-1}) is similar to that for lateral diffusion (33–80 kJ mol^{-1}; Kuo & Wade, 1979), indicating that a similar energy barrier is operative in both cases which can be identified with the formation of a lattice vacancy in the bilayer.

Physiological Significance. The aqueous diffusion mechanism appears to be a general phenomenon applicable to all lipids. Thus, in addition to cholesterol, phospholipids, and a variety of fluorescent lipid molecules which exchange between vesicles or lipoproteins via the aqueous phase, PC exchange catalyzed by the phosphatidylcholine exchange protein also operates according to an aqueous diffusion mechanism where the aqueous phase species is PC bound to exchange protein (Wirtz, 1974). These proteins serve to increase the rate of exchange of sparingly soluble lipids such as PC and cholesterol esters by increasing their effective aqueous phase solubility. Interestingly, the flux of PC and cholesterol ester in the presence of lipid exchange proteins is similar to that of cholesterol in a protein-free system (Table III). Comparable fluxes have been observed for cholesterol transfer from tissue culture cells to PC vesicles (Phillips et al., 1980) and across the rat aorta intimal surface (Dayton & Hashimoto, 1966). These observations suggest that an exchange protein is required in vivo for sparingly soluble lipids, but not for cholesterol, since the uncatalyzed flux of this more soluble molecule is sufficient for physiological purposes.

The aqueous diffusion mechanism is also expected to be important for cholesterol transfer in many biological systems. Since both mass transfer and mass exchange of lipid can be described by the same rate constant (Lange & D'Alessandro, 1977; L. R. McLean and M. C. Phillips, unpublished results), these processes differ only in the initial distribution of lipid between the donor and acceptor particles. The mechanism is the same in both cases. Mass transfer occurs when the activity of exchangeable lipid is greater in the donor particles than in the acceptor particles; mass exchange operates at equilibrium when these activities are identical.

Table III: Interfacial Flux of Lipids in the Absence and the Presence of Lipid Exchange Protein[a]

systems	lipid transferred	exchange protein	J (molecules/10 nm^2 h^{-1})
vesicle–vesicle[b]	cholesterol	–	1.15
mitochondria–microsomes[c]	phosphatidylcholine	+	0.91
LDL–HDL[d]	cholesterol ester	+	0.72

[a] Fluxes (J) were calculated as described under Methods. [b] $k = 8.9 \times 10^{-5}$ s^{-1}, $c = 3.0 \times 10^{-5}$ M, $A/V = 5.0 \times 10^{2}$ cm^2 L^{-1}. [c] $kcV = 1 \times 10^{-11}$ mol s^{-1} (Wirtz et al., 1972), $A = 2.4 \times 10^{3}$ cm^2 [using data from Spiro & McKibbin (1956) and deDuve (1963–1964) on mitochondria]. [d] $kcV = 3.4 \times 10^{-13}$ mol s^{-1} (Barter & Jones, 1980), $A = 5.3 \times 10^{2}$ cm^2 [using data from Kezdy (1978) on LDL].

Presently the factors which determine the kinetics of cholesterol desorption from model membranes are under investigation. Preliminary results indicate that uncharged donor vesicles exchange cholesterol at a rate comparable to that for charged vesicles. Further research will investigate the effects of cholesterol/phospholipid ratio, phospholipid acyl chain length, and vesicle curvature on the rate and activation energy of cholesterol exchange and transfer.

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Interaction of the Activated Cytoplasmic Glucocorticoid Hormone Receptor Complex with the Nuclear Envelope†

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ABSTRACT: Highly purified activated cytoplasmic glucocorticoid hormone receptor binds with high affinity to sites in the nuclear envelope. Nuclear envelope fragments can be isolated from purified chromatin. They bind activated cytoplasmic glucocorticoid receptor with the same equilibrium constant as nuclear envelopes. The presence of envelope components in chromatin is confirmed by the virtual identity of the gel electrophoretic glycoprotein pattern of nuclear envelope, chromatin nonhistones, and nuclear envelope fragments from chromatin.

It is well established that under "in vitro" and "in vivo" conditions steroid hormones including the glucocorticoids initiate increased transcription (Young et al., 1974; Jensen & de Sombre, 1972; O'Malley et al., 1977). There are good indications that such inductions of messenger ribonucleic acid (mRNA) synthesis in general are accompanied or preceded by enzymatic differential modifications of nuclear proteins (Stein et al., 1974). The initiation of the enzymatic nuclear protein modification which in turn may render the DNA transcribable must be preceded by the interaction of the inducing hormone with a suitable macromolecule in the nucleus. It is generally accepted that prior to its localization in the nucleus steroid hormones are bound by cytoplasmic receptors which in turn interact with a nuclear acceptor site (Spelsberg, 1974). A considerable amount of literature has been generated over the years, purporting that the nonhistone chromosomal proteins contain these acceptor sites, thus exerting a controlling function on the transcriptional activity of the genome (O'Malley et al., 1977). However, a number of authors have leveled criticism at the criteria of purity of nonhistone prep-

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